C PP-2002-0308

Plant Physiol. (1992) 99, 74–80
0032-0889/92/99/0074/07/$01.00/0

Received for publication July 24, 1991
Accepted November 26, 1991

Immunodetection and Identification of N^6-(o-Hydroxybenzylamino)Purine as a Naturally Occurring Cytokinin in *Populus* × *canadensis* Moench cv *Robusta* Leaves[1]

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ABSTRACT

A highly specific and sensitive enzyme-linked immunosorbent assay (ELISA) was developed for 9-β-D-ribofuranosyl-N^6-(o-hydroxybenzylamino)purine [(oOH)[9R]BAP] and structurally related cytokinins. As little as 3 femtomoles of the compound could be detected by this method. Cross-reactivity studies demonstrated the specificity of four polyclonal antibodies for (oOH)[9R]BAP and its free base in preference to a range of natural cytokinins and other purines. After evaluating the method by internal standardization employing [2-^{3}H](oOH)[9R]BAP of high specific radioactivity as recovery marker by dilution analyses and by immunohistograms, it was possible to apply ELISA to quantify (oOH)[9R]BAP in plant extracts. In addition to (oOH)[9R]BAP, an unknown cytokinin reacting with the same antibody was detected in high performance liquid chromatography-fractionated extracts of mature *Populus* × *canadensis* Moench cv *Robusta*. The structure of the new compound was determined by gas chromatography-mass spectrometry and finally confirmed by synthesis as N^6-(o-hydroxybenzylamino)purine.

Most natural as well as many synthetic cytokinins contain an isoprenoid side chain attached to the N^6-position of the adenine (14). Cytokinins with an aromatic ring substituting at N^6 were supposed to occur only sporadically in a few plant species (13, 14). Horgan *et al.* (9, 10) isolated the first cytokinin of that type from fully expanded *Populus* × *Robusta* Schneid leaves and identified it as N^6-(o-hydroxybenzylamino)-9-β-D-ribofuranosylpurine. Later, this compound was found, together with its 2-methylthioglucofuranosyl derivative (4, 5), in the fruits of *Zantedeschia aethiopica*. Recent identification of [9R]BAP (Table I) in an old *Pimpinella anisum* L. cell culture (8) supports the idea that this compound may be the precursor of the hydroxylated aromatic cytokinins. As suggested from the biological activities, the hydroxylation of the benzyl ring of [9R]BAP in the o-position may represent a deactivation step, whereas the hydroxylation in the *m*-position can generate the opposite effect (11). For accurate and fast analysis of [9R]BAP and its metabolites, we developed an analytical procedure that is based on the testing of the immunoreactivity in HPLC-fractionated extracts (21, 22). Using this approach, the free base N^6-(o-hydroxybenzylamino)purine was identified in *Populus* × *canadensis* Moench cv *Robusta* leaves as a naturally occurring cytokinin. We also report the preparation and characterization of the ELISA for the determination of (oOH)[9R]BAP in plant extracts. Preliminary data about this ELISA have already been published (20, 21).

[1] Research was supported by the Czechoslovak Academy of Sciences and by a grant from the Deutsche Forschungsgemeinschaft within the scope of SFB 137.

MATERIALS AND METHODS

Plant Material

Fully expanded leaves were collected from 1-year-old shoots of *Populus* × *canadensis* Moench cv *Robusta* in the field. Detached leaves were weighted and immediately plunged into liquid nitrogen.

Cytokinins and Other Chemicals

[9G]Z, [9R-5'P]Z, (OG)Z, (OG)[9R]Z, (diH)[9G]Z, (diHOG)[9R]Z, (diHOG)Z, and (diH)[9R-5'P]Z were purchased from Apex (Oxford, UK). (oOH)BAP was prepared by acid hydrolysis of (oOH)[9R]BAP in 1 N TFA and purified by reversed phase HPLC as described earlier (21). [2-^{3}H](oOH)[9R]BAP (0.52 TBq/mmol) was kindly supplied by Dr. J. Hanus, Institute of Nuclear Biology and Radiochemistry, Prague, Czechoslovakia. Ostsorb DEAE-cellulose, cellulose phosphate, Separon SGX C_{18} columns were from Tessek (Prague, Czechoslovakia); acetonitrile for chromatography was purchased from Merck. Triethylammonium hydrogen carbonate was from Serva; N-methyl-N-(TMS)trifluoro acetamide was purchased from Sigma. All other chemicals were from sources described elsewhere (21) or purchased from Lachema (Brno, Czechoslovakia).

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Table I. *Abbreviations Used in this Paper*

BAP, N^6-benzylaminopurine
(mOH)BAP, N^6-(*m*-hydroxybenzylamino)purine
(oOH)BAP, N^6-(*o*-hydroxybenzylamino)purine
[9R]BAP, 9-β-D-ribofuranosyl-BAP
(mOH)[9R]BAP, 9-β-D-ribofuranosyl-(mOH)BAP
(oOH)[9R]BAP, 9-β-D-ribofuranosyl-(oOH)BAP
(diH)Z, dihydrozeatin
(diH)[9G]Z, 9-β-D-glucopyranosyl-(diH)Z
(diH OG)Z, O-β-D-glucopyranosyl-(diH)Z
(diH)[9R]Z, dihydrozeatin riboside
(diH)[9R-5'P]Z, dihydrozeatin riboside 5'-monophosphate
(diH OG)[9R]Z, O-β-D-glucopyranosyl-(diH)[9R]Z
iP, N^6-(Δ^2-isopentanyl)adenine
[9R]iP, 9-β-D-ribofuranosyl-iP
K, kinetin
[9R]K, 9-β-D-ribofuranosyl-K
Z, *trans*-zeatin
(*cis*)Z, *cis*-zeatin
[9G]Z, 9-β-D-glucopyranosyl-Z
(OG)Z, O-β-D-glucopyranosyl-Z
[9R]Z, *trans*-zeatin riboside
[9R-5'P]Z, zeatin riboside 5'-monophosphate
(OG)[9R]Z, O-β-D-glucopyranosyl-[9R]Z
TMS, trimethylsilyl
TBS, Tris-buffered saline

Extraction and Purification of the Cytokinin Fraction

Cytokinins were extracted from fully expanded *P.* × *Robusta* leaves (1 g of fresh leaf material for immunodetection, 100 g for identification) after homogenization in 80% (v/v) methanol. Extracts were purified by butanol extraction followed by cation-exchange chromatography on cellulose phosphate and by combined DEAE-cellulose-reversed phase chromatography (1, 15, 21). [2-^{2}H](oOH)[9R]BAP (approximately 50,000 cpm) was used as internal standard for estimating the recovery at each purification step.

HPLC

For the immunodetection, cytokinins were fractionated on a Separon SGX C_{18} column (250 × 4 mm, particle size, 7 μm). The column was eluted with a gradient of methanol (32–56%) in 40 mM acetic acid, adjusted to pH 3.4 with distilled triethylamine (21). Fractions of 0.5 mL were collected, evaporated *in vacuo* to dryness, and dissolved in 50 μL DMSO and 950 μL TBS buffer (50 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.02% [w/v] NaN_3, pH 7.5). Each fraction was analyzed by ELISA.

For GC-MS, the partially purified and filtered extracts were subjected to HPLC on a 10-μm Separon SGX C_{18} column (250 × 8 mm). Cytokinins were eluted at a flow rate of 2 mL/min using the gradient described above. The effluent was evaporated to dryness, tested by the ELISA, and fractions containing immunoreactive material were rechromatographed twice on a Hypersil ODS column (250 × 4.6 mm, 5 μm, Shandon, Cheshire, UK). The column was eluted at a flow rate of 1.2 mL/min with acetonitrile and 5 mM triethylammonium hydrogen carbonate buffer (adjusted to pH 6.5 with distilled acetic acid) using the following gradient of acetonitrile: 15% (v/v) for 10 min; 15 to 18% (v/v) over 5 min; 18 to 18.5% (v/v) over 50 min. Subsequently, the column was washed with 100% acetonitrile for 10 min and regenerated with 15% (v/v) acetonitrile in triethylammonium bicarbonate buffer. Using this procedure, cytokinin retention times were 43.8 min for (oOH)BAP and 48.88 min for (oOH)[9R]BAP. The recovery of the cytokinins was followed with [2-^{2}H](oOH)[9R]BAP. Ten-microliter aliquot volumes of each fraction from the HPLC run were investigated by scintillation counting and in duplicate by ELISA. The (oOH)[9R]BAP-immunoreactive fractions were dried using a Speed Vac Concentrator (model SVC 100H, Savant, New York). Fractions containing (oOH)[9R]BAP and its free base were dissolved in 5 μL tetrahydrofuran (20–200 μg/μL) mixed with 10 μL of *N*-methyl-*N*-(TMS)trifluoro acetamide and kept at 60°C for 1 h. Aliquot volumes of 1 μL of the supernatant were injected into the gas chromatograph.

GC-MS

A Varian gas chromatograph, model 3700 (Bremen, FRG) equipped with an injector for split-splitless injection (270°C) was directly connected to the source of the MS. The GC was furnished with a 30 m × 0.3 mm (i.d.) bonded phase (DB-1)-fused silica capillary column (J&W Scientific, Folsom, CA); the temperature program ranged from 100 to 300°C at a rate of 2°C/min, and helium (2 mL/min) was used as carrier gas. GC-MS analysis was carried out using a Finnigan MAT 312 spectrometer with an inverse Nier-Johnson geometry and a combined electron impact/chemical ionization ion source. Electron impact spectra were determined at an ionization energy of 70 eV. The GC-MS was directly coupled to a Finnigan MAT SS 300 data system.

Preparation of Cytokinin Conjugate and Enzyme Tracer

Following the procedure of Eberle *et al.* (6), (oOH)[9R]BAP was coupled via periodate-oxidized *vic*-hydroxy groups of the ribose to the free amino groups of BSA or alkaline phosphatase (3000 units/mg). A coupling ratio of 11 mol cytokinin/mol of BSA was determined by spectrophotometry.

Immunization Protocol

The hapten conjugate (1.0 mg) was dissolved in 2.5 mL PBS buffer (50 mM Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl, pH 7.2) and mixed with an equal volume of Freund's complete adjuvant. Four rabbits were immunized, each by four injections of freshly prepared antigen emulsion (0.4 mL equally divided into its foot pads), followed by multiple-site intradermal boostering with portions of about 100 μL of emulsion at days 0, 7, 28, 42, 72, 102, and 132. Blood was collected on the seventh day after the last injection and the immunoglobulin G fraction was precipitated with 50% saturated ammonium sulfate, lyophilized, and stored at −20°C.

ELISA

Assays of (oOH)[9R]BAP-type cytokinins were carried out on KOH-I-NOOR flat bottom plates (České Budějovice,

Czechoslovakia) using a modification of the ELISA described by Weiler *et al.* (27) for auxin. The wells were filled with 150 μL 50 mM $NaHCO_3$ (pH 9.6) containing 5 μg/mL immunoglobulin G, incubated overnight at 4°C for binding, and then washed twice with water to remove unbound antibody. To saturate remaining protein binding sites, the wells were filled with 200 μL of 0.02% (w/v) BSA in TBS buffer and incubated for 60 min at 4°C. After being rinsed with water, the coated wells were filled with 50 μL of TBS, 50 μL of sample or standard in TBS, and 50 μL of the cytokinin-alkaline phosphatase conjugate (0.1 μg/mL) in 0.02% (w/v) BSA in TBS and the plates were incubated at 4°C for 60 min. Unbound conjugates were removed by rinsing the plates four times with TBS buffer. The activity of bound alkaline phosphatase was determined using *p*-nitrophenylphosphate as a substrate (150 μL, 1 mg/mL in 50 mM $NaHCO_3$, pH 9.6, incubation for 60 min at 37°C). The reaction was terminated with 50 μL of 3 N KOH and the absorbance at 405 nm was read. Calibration of the ELISA was performed as described previously (21). Calculations of results were carried out as described by Weiler *et al.* (27). Sigmoidal standard curves for (oOH)[9R]BAP, cross-reacting compounds, dilution analysis, and internal standardization were linearized by the following transformation: logit B/Bo = ln(B/Bo)/(100-B/Bo) (see Fig. 1).

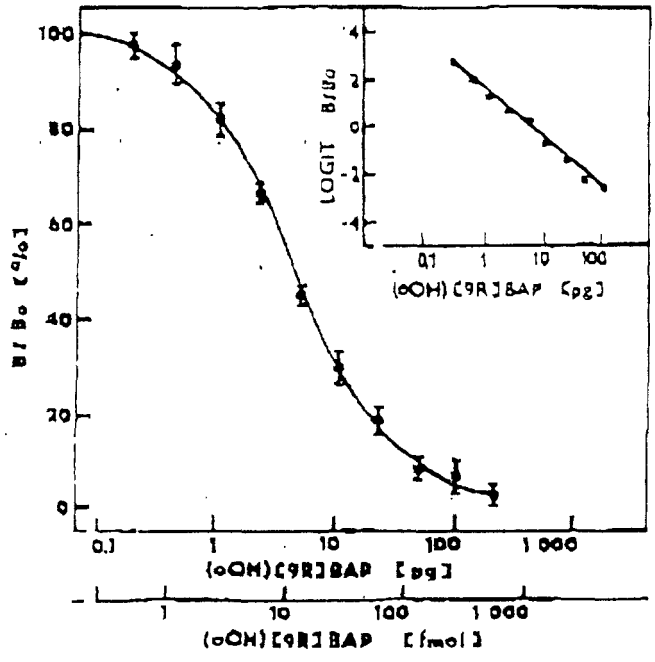

Figure 1. Standard curve for the (oOH)[9R]BAP ELISA and linearized logit/log plot of the same data (inset). Bars indicate SE (n = 20). B and Bo represent binding of alkaline phosphatase tracer in the presence and the absence of (oOH)[9R]BAP standard, respectively.

RESULTS

Sensitivity and Specificity of the (oOH)[9R]BAP ELISA

Antisera were raised in rabbits against the (oOH)[9R]BAP-BSA conjugate that had been prepared by the periodate method. Using the immunization protocol described above, antisera of high quality and comparable sensitivity and specificity were obtained (Table II). Because of the lowest degree of overall cross-reactivity, Ab 165 was routinely used for cytokinin analysis by ELISA. A typical standard curve obtained with Ab 165 and (oOH)[9R]BAP is shown in Figure 1. The inset shows the linearized curve providing a measuring range between 2.5 and 670 fmol/assay. Because as little as 3.05 fmol (1.14 pg) of (oOH)[9R]BAP could be detected, the assay was very sensitive. The midrange value (amount of antigen required for 50% inhibition) was 103 fmol for this cytokinin. Unspecific binding (binding in the presence of an excess of (oOH)[9R]BAP:313 pmol) was less than 3.5%. The coefficient of variation for the duplicate determination of standard B/Bo values within the measuring range was less than 4.0% in all of the ELISAs.

Specificity of polyclonal antibodies was determined by cross-reactivity studies (Table II). Cytokinins and other compounds that produced molar cross-reactions lower than 0.01% are not presented in Table II. Adenine, adenosine, inosine, and all natural isoprenoid cytokinins of the zeatin and dihydrozeatin group such as Z, [9R]Z, [9R-5'P]Z, (OG)[9R]Z, (OG)Z, [9G]Z, (diH)Z, (diH)[9R]Z, (diH)[9R-5'P]Z, (diH)[9G]Z, (diHOG)Z, (diHOG)[9R]Z, and the nonpurine cytokinin *N,N'*-diphenylurea, exhibited almost zero cross-

Table II. *Molar Cross-Reactivities of Several Cytokinins with (oOH)[9R]BAP Antibodies*

Data presented are expressed as percentage ratio of molar concentrations of (oOH)[9R]BAP and competitor producing 50% inhibition.

Compound	Antibody			
	164	165	166	167
(oOH)[9R]BAP	100	100	100	100
(oOH)BAP	50.50	24.61	11.11	31.82
(mOH)[9R]BAP	0.27	0.04	0.32	0.03
(mOH)BAP	0.20	0.02	0.20	0.02
[9R]BAP	0.87	0.09	1.50	0.18
BAP	0.39	0.08	0.60	0.14
[9R]IP	0.15	0.03	0.12	0.14
IP	0.09	0.04	0.08	0.08
[9R]K	0.10	0.04	0.20	0.13
K	0.06	0.03	0.10	0.10

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reactivity. These compounds did not react even when present at 1 nmol/assay. [9R]iP and its free base showed at most a slight cross-reactivity. The same holds for the synthetic cytokinins kinetin and kinetin riboside. Interestingly, antibodies did not significantly bind cytokinins with unsubstituted or *m*-hydroxylated benzyl rings. The cross-reactivity data suggest that the *o*-position of the hydroxy group on the benzyl ring is one of the crucial requirements for immunoreactivity. Thus, hydroxylation in the *m*-position results in almost complete loss of immunoreactivity with (oOH)[9R]BAP antibodies. The low cross-reactivity with all tested cytokinins shows the high specificity of the four individually produced antibodies. On the other hand, the specificity with respect to the ribose moiety was low, as indicated by high immunoreactivity with the free base (oOH)BAP in all four ELISAs; its cross-reactivity was in the range between 11.1 and 50.5%.

Detection and Identification of (oOH)BAP in Poplar Leaves

The cross-reactivity studies showed that the ELISA used in this study provides a sensitive and specific assay for (oOH)[9R]BAP-type cytokinins. It was used to examine the occurrence of cytokinins structurally related to (oOH)[9R]BAP in plant tissues. Because the riboside has been identified in *Populus* × *Robusta* Schneid leaves (9, 10), this plant material (under the present scientific name *Populus* × *canadensis* Moench cv *Robusta*) was further investigated by the (oOH)[9R]BAP ELISA. The leaf extract was purified by butanol extraction and chromatography on cellulose phosphate, DEAE-cellulose, and octadecylsilica columns prior to HLPC and each fraction was assayed by ELISA. Approximately 95% of the immunoreactive material applied to the HPLC column was recovered in two peaks (Fig. 2). One peak coeluted with (oOH)[9R]BAP whereas the other, according to its retention time, could not be ascribed to any known cytokinin. With respect to its chromatographic behavior, this compound was tentatively interpreted as the free base (oOH)BAP. By acidic hydrolysis of (oOH)[9R]BAP, the free base was prepared and, upon HPLC, it coeluted with the unknown substance.

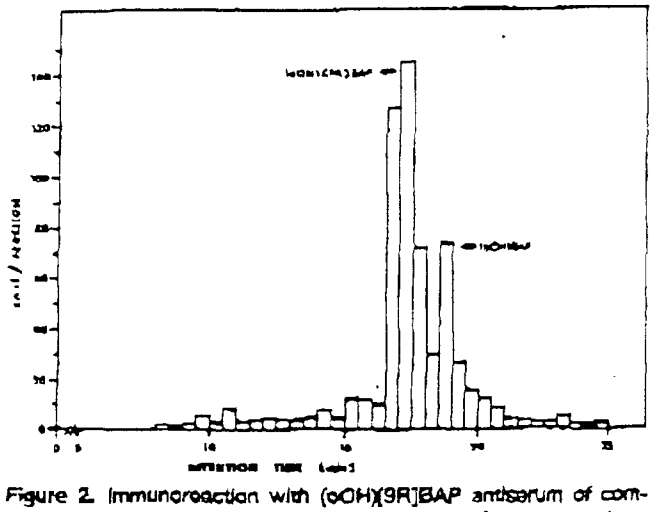

Figure 2. Immunoreaction with (oOH)[9R]BAP antiserum of compounds present in HPLC fractions of partially purified extracts from mature *P.* × *canadensis* Moench cv *Robusta* leaves. Chromatographic details and HPLC separation of different cytokinins have been described previously (21). Retention times of (oOH)[9R]BAP and (oOH)BAP standards are indicated by arrows.

ELISA was used in the purification procedure for isolation of substantial amounts of (oOH)BAP and its riboside for GC-MS analysis. Validation of the immunoassay data was achieved by serial dilution of the analyzed extracts and their HPLC fractionation with or without standard amounts of (oOH)BAP and (oOH)[9R]BAP. The diluted extracts analyzed by ELISA at different purification steps always produced parallel curves (Fig. 3A). Similarly, internal standardization of either crude, partially purified, or HPLC-fractionated extracts resulted in straight lines parallel to the standard curve (Fig. 3B). The HPLC fractions contained small amounts of immunoreactive material other than (oOH)[9R]BAP and its free base, which could not be ascribed to any of the cytokinin standards on the retention time basis (Fig. 2). The specificity of ELISA suggested that the unknown compounds may be structurally related to (oOH)[9R]BAP.

To confirm the presence of (oOH)BAP in the immunoreactive peak, the respective HPLC fraction was trimethylsilylated and subjected to GC-MS analysis. The TMS derivative produced a mass spectrum (Fig. 4) that was identical to that of an authentic standard. In addition the molecular ion of m/z 313 shows the expected mass of (oOH)BAP containing only 1 TMS group. The presence of fragment ions at m/z 135, 120, and 119 is indicative of an adenine fragment. Shannon and Letham (18) suggested ions of m/z 148 and 149 as a characteristic feature of an adenine substituted with a $-CH_2-$ group at N^6. Hence, mass fragments at m/z 163, 179, and 194 presumably represent the remaining fragments of the side chain that must be of an aromatic nature. The loss of a trimethylsilylated hydroxyl group (Δm 89) from the molecular ion indicated the presence of a hydroxyl group on the benzyl ring (m/z 224). The m/z 385 ion arises from a di-TMS derivative with the additional TMS group attached to N^9 of adenine. Hence, the coincidence of the mass spectra of an authentic test substance and the unknown compound, as well as the characteristic mass and fragments, provide evidence for the chemical structure of the compound as N^6-(*o*-hydroxybenzylamino)purine.

Quantification of (oOH)[9R]BAP and (oOH)BAP in the Leaf Extracts

The content of (oOH)[9R]BAP and its free base/g fresh weight of *P.* × *canadensis* Moench cv *Robusta* leaf tissues was estimated by the ELISA using an internal standard [2-^{3}H](oOH)[9R]BAP (78% yield). The level of (oOH)[9R]BAP determined from five duplicate estimates was found to be 125.5 ± 13.4 ng/g fresh weight. The respective correction was made for (oOH)BAP on the basis of its molar cross-reactivity in the (oOH)[9R]BAP ELISA. The content of this cytokinin in mature leaves, as estimated by internal standardization, was 134 ± 18.3 ng/g fresh weight. The fraction containing this cytokinin exhibited the following UV spectral characteristics (λ_{max}): 80% (v/v) ethanol, 267.0; 0.02 *N*

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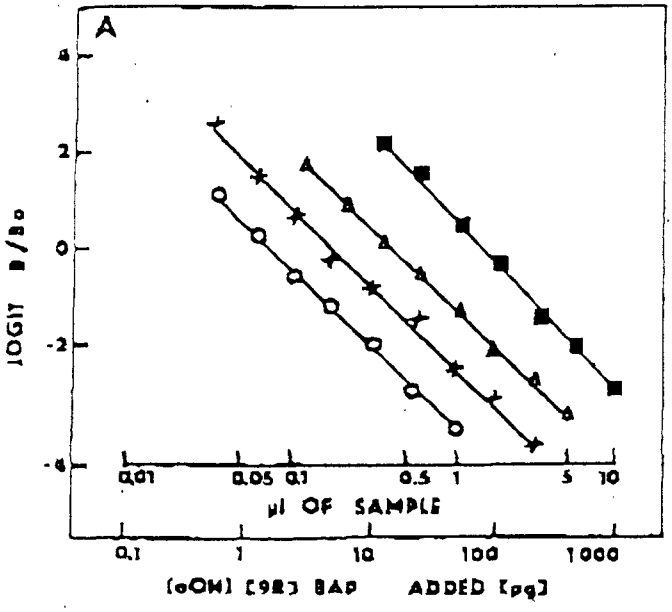

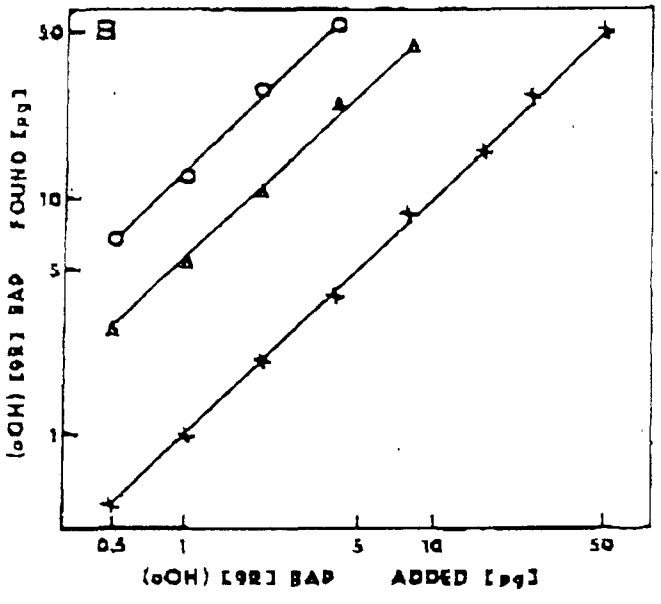

Figure 3. A, Logit transformation of ELISA standard curve for (oOH)[9R]BAP (×) and sample dilution curves for a *P.* × *canadensis* Moench cv *Robusta* extract at different purification steps: (○), crude extract; (△) ammonia eluate from cellulose phosphate; (■) HPLC fractions containing (oOH)[9R]BAP. B, Logit transformation of ELISA data using calibration standards (×) or a fixed amount (0.5 µL) of crude (○), or of partially purified (DEAE-cellulose-octadecyl silica chromatography) (△) extract of *P.* × *canadensis* Moench cv *Robusta* leaves after addition of different amounts of unlabeled (oOH)[9R]BAP.

NH_4OH in 80% (v/v) ethanol, 274.5; 0.1 N CH_3COOH, 273.5, which are indicative of a N^6-monosubstituted adenine (12).

DISCUSSION

Allowing quantification of as little as 3 fmol/assay, the ELISA described here is 1 order of magnitude more sensitive than comparable ELISAs developed for isoprenoid cytokinins (26). Similar sensitivity has been reported only with the solid-phase ELISA for [9R]iP using a detection system based on the high affinity between avidin and biotin (19). Our assay is highly specific for the N^6-substituent of (oOH)BAP and does not cross-react with the corresponding *m*-hydroxy compound or the unhydroxylated BAP. This strong discrimination was found with all four antisera and, hence, must be typical of this kind of antibody. High specificity for any substituent at N^6 was consistently found when the cytokinin hapten and protein (BSA) were conjugated via a spacer such as ribose. Nevertheless, the selectivity, as well as the high sensitivity of the ELISA described here, suggest that aromatic cytokinins provide better epitopes for antibody recognition than isoprenoid cytokinins (21, 22).

The high cross-reactivity between the antibodies raised against the riboside and the (oOH)BAP suggests that alterations at the adenine moiety have less impact on the intensity of the immunoreaction than changes of the N^6-substituent. Hence, in addition to the ribosids and the free base, other (oOH)BAP derivatives (*e.g.* the 5′-monophosphate or N^9-glucosides or amino acid conjugates) may also cross-react with antibodies and thus presumably can be measured with the (oOH)[9R]BAP ELISA (14) if present in the same HPLC fraction. Unfortunately, such compounds were not available. On the other hand, glycosylation of the o-hydroxy group could change the hapten dramatically, rendering a cross-reaction with (oOH)[9R]BAP antisera very unlikely.

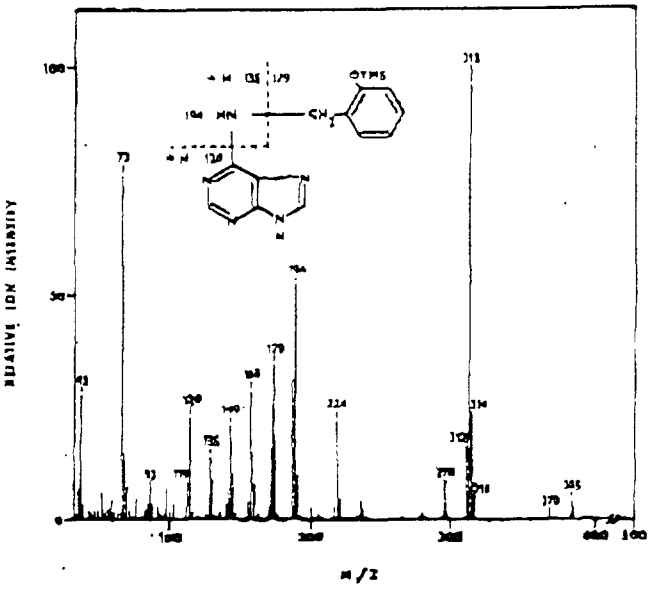

Figure 4. Mass spectrum of TMS derivative of the unknown aromatic cytokinin.

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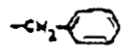
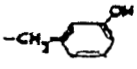
SUBSTITUENT R_1	R_2	R_3	ABBREVIATION	REFERENCE
	H	H	BAP	(16, 17, 22)
	H	Rib	[illegible]	(8, 16, 17, 22)
	H	Rib-5'-P	[illegible]	(16)
	H	Glc	[illegible]	(17)
	H	H	(oOH)BAP	(p.c.)[a]
	H	Rib	(oOH)[illegible]	(4, 9, 10)
	CH_3S	Glc	(oOH)[illegible]	(5)
	H	H	(mOH)BAP	(22, 23)
	H	Rib	(mOH)[illegible]	(22, 23)

[a] p.c. = personal communication

Figure 5. Naturally occurring aromatic cytokinins identified in plant species.

As a consequence of the high affinity between antigene and antibody, the ELISA described here is less prone to nonspecific interference with other compounds of a plant extract than other analytical procedures. This was demonstrated by validating the assay in a series of internal quality controls, including sample dilution analysis (Fig. 3A), internal standardization (Fig. 3B), and HPLC fractionation (Fig. 2). Even when the crude leaf extract was investigated, the ELISA characteristic was parallel to that produced with the pure cytokinin (Fig. 3A). Low interference with contaminants appears to be typical of most of the cytokinin ELISAs (1, 3, 6). With respect to the riboside of (oOH)BAP in poplar leaves, quantification by ELISA is in fair agreement with the results obtained previously with the soybean callus bioassay (10). However, our data support the findings of several laboratories that bioassays show somewhat lower cytokinin concentrations than GC-MS, radioimmunoassay, or ELISA (1). The reason for the apparently smaller amounts determined with the bioassay may be attributed to an eventual interference with inhibitory compounds in the extracts (7).

The identification of (oOH)BAP in *P.* × *canadensis* leaves (Fig. 4), together with the detection of several immunoreactive compounds in the HPLC fractions by ELISAs for (mOH)[9R]BAP and [9R]BAP (22), show that aromatic cytokinins presumably will occur in many more plant species than has been previously supposed. The structures of aromatic cytokinins identified so far in plant tissues are shown in Figure 5. Notably, BAP and its analogs constitute a distinct class of cytokinins occurring naturally in plants. Further studies of the chemical structure of compounds of that class are in progress.

ACKNOWLEDGMENTS

The authors wish to thank H. Martinková for excellent technical assistance. We also wish to thank Dr. J. Rainer and M. Glaessner from the Zentrale Analytik of the University of Bayreuth for their help with the GC-MS.

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